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Protozoan Cysts Act as a Survival Niche and Protective Shelter for Foodborne Pathogenic Bacteria

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The production of cysts, an integral part of the life cycle of many free-living protozoa, allows these organisms to survive adverse environmental conditions. Given the prevalence of free-living protozoa in food-related environments, it is hypothesized that these organisms play an important yet currently underinvestigated role in the epidemiology of foodborne pathogenic bacteria. Intracystic bacterial survival is highly relevant, as this would allow bacteria to survive the stringent cleaning and disinfection measures applied in food-related environments. The present study shows that strains of widespread and important foodborne bacteria (*Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica*, and *Listeria monocytogenes*) survive inside cysts of the ubiquitous amoeba *Acanthamoeba castellanii*, even when exposed to either antibiotic treatment (100 μg/ml gentamicin) or highly acidic conditions (pH 0.2) and resume active growth in broth media following excystment. Strain- and species-specific differences in survival periods were observed, with *Salmonella enterica* surviving up to 3 weeks inside amoebal cysts. Up to 53% of the cysts were infected with pathogenic bacteria, which were located in the cyst cytosol. Our study suggests that the role of free-living protozoa and especially their cysts in the persistence and epidemiology of foodborne bacterial pathogens in food-related environments may be much more important than hitherto assumed.

Foodborne pathogenic bacteria are a major cause of foodborne illness and have important implications for human public health along with economic consequences (1). Despite thorough disinfection protocols and hygiene monitoring during food production and processing, pathogenic bacteria often persist in food-related environments and on food, suggesting that our knowledge about the transmission routes and epidemiology of foodborne pathogenic bacteria is still incomplete.

Recent studies have shown that bacteria can benefit from intracellular associations with free-living protozoa (FLP) (2, 3), heterotrophic eukaryotic microorganisms that are common in natural aquatic and terrestrial ecosystems (4). Although FLP feed on bacteria, some bacteria resist digestion. These so-called "digestion-resistant bacteria" can survive and even grow inside their FLP hosts (5). These hosts thus effectively act as a reservoir, shelter, and vector for the bacteria and can as such play an important role in their ecology (3, 6). Intracellular association with FLP has also been demonstrated for human-pathogenic bacteria (7, 8), including food-related pathogens (e.g., see references 5, 9, and 10). As FLP have been isolated from diverse food-related habitats, such as broiler houses (11–13), meat cutting plants (14), domestic refrigerators (15), and vegetables (16, 17), this suggests that FLP may be implicated in the epidemiology of foodborne pathogens.

Many FLP have two life cycle stages: the trophozoite and the dormant cyst. The former is the actively feeding stage, preying on bacteria, algae, viruses, yeast, and organic particles by phago- and pinocytosis (18). Encystment (i.e., conversion from trophozoite to cyst) is triggered by adverse environmental conditions, such as food shortage, hyper- or hypo-osmolarity, temperature, and pH extremes (18). Cysts usually possess a thick, often double or multilayered protective wall, consisting of lipids, (glyco)proteins, and carbohydrates like chitin and cellulose. This protects the protozoon against unfavorable environmental conditions, such as freezing (19), gamma and UV radiation (20), and chemicals used for disinfection in health care settings (21) and drinking water production (22, 23). Some cysts can withstand desiccation for more than 20 years (24). Under favorable conditions, excystment (i.e., reconversion into trophozoites) takes place.

To date, most studies on interactions between FLP and bacteria (including pathogenic bacteria) have focused on the trophozoite stage. In contrast, little is known about bacterial association with the cyst forms, which, given their high tolerance for adverse environmental conditions and hence also high dispersal capacity (18), are especially relevant from an ecological and epidemiological point of view. It is expected that FLP cysts are even more effective as a shelter and vector for internalized bacteria than the trophozoites. It has indeed been shown that some internalized digestion-resistant bacteria, including human pathogens, can survive the encystment process and may use the cysts as a shelter against harsh environmental conditions (19, 25). When environmental conditions become favorable, excystment occurs and internalized bacteria are released (or trigger their release), allowing these bacteria to colonize new habitats (26).

As FLP can act as a vector and shelter, there is a growing concern that cysts may play a role in the contamination and persistence of pathogenic bacteria in food-related environments. Cysts may thus enable internalized foodborne pathogens to survive physical and chemical cleaning and disinfection methods (21, 23).

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To date, long-term intracystic survival of foodborne pathogenic bacteria isolated from food had never been assessed. In order to evaluate the role of FLP in the persistence of foodborne bacterial pathogens, more information is needed on intracellular bacterial survival during the en- and excystment processes and on survival duration inside the FLP cysts. To this end, two strains each of the five most frequently reported foodborne pathogenic bacteria with a considerable impact on public health and the food industry (27) were selected: *Campylobacter jejuni*, *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica*, and *Listeria monocytogenes*. The amoeba *Acanthamoeba castellanii* was used as an FLP organism, as it is frequently detected in food-related environments (11, 15). In the present study, we (i) investigated the uptake/invasion efficiency of these foodborne bacteria by *A. castellanii* trophozoites, (ii) determined the long-term survival capacities of these bacteria inside amoebal cysts using an uniform experimental design, (iii) evaluated the impact of bacteria on protozoan encystment, and (iv) assessed the exact location of these bacteria inside the cysts. To date, survival inside *Acanthamoeba* cysts has been reported for *Mycobacterium* spp. (19, 28), *Francisella tularensis* (26), *Legionella* spp. (25), *Simkania negevensis* (29), *Escherichia coli* (30, 31), *Vibrio* spp. (32), *Enterobacter aerogenes*, *Aeromonas hydrophila* (33), and some obligate endosymbionts, among which is *Protochlamydia amoebophila* (34, 35). The present study specifically addresses the association mode and survival duration of the most common foodborne pathogens inside FLP cysts.

MATERIALS AND METHODS

Amoebal strain and culture conditions. *Acanthamoeba castellanii* (ATCC 30234; American Type Culture Collection) was grown axenically in proteose-peptone-yeast extract-glucose medium (PYG) (ATCC recipe; www.lgcstandards-atcc.org) at 25°C in 75-cm^2 tissue culture flasks. Light microscopic observations and plating of culture samples on plate count agar (PCA) (Bio-Rad, Hercules, CA), which were incubated at 30°C for 48 h, were performed to verify the axenicity of the cultures. After 3.5 days, the amoebae formed a confluent monolayer and were harvested by tapping flasks and subsequent centrifugation of the cell suspensions (300 × *g* for 5 min). Amoebae were washed with Page's amoeba saline (PAS) (ATCC recipe) and suspended in high-saline (HS) buffer (0.1 M KCl, 8 mM $MgSO_4 \cdot 7H_2O$, 0.02 M Tris, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$ [pH 9]), the medium that will also be used in further experiments to induce encystment (28). The number of viable trophozoites at the start of each experiment was determined by trypan blue exclusion assays (10) using a Füchs-Rosenthal counting chamber (Blaubrand, Wertheim, Germany) and adjusted to 5×10^5 viable trophozoites/ml HS buffer.

Bacterial strains and culture conditions. Throughout this study, 10 strains belonging to five foodborne pathogenic bacterial species were used: a *C. jejuni* isolate from chicken sausage, displaying low invasiveness properties toward Caco2 cells, and a high-invasiveness *C. jejuni* isolate from marinated chicken wings (9); *S. enterica* serotypes Typhimurium and Enteritidis, both isolated from pig carcasses; enterohemorrhagic (EHEC) *E. coli* biotypes O:157 and O:26, isolated from cattle carcasses; *Yersinia enterocolitica* bioserotype 4/O:3, isolated from pig carcass, and bioserotype 2/O:9 from minced pork meat (10), both carrying the virulence plasmid (pYV); and *L. monocytogenes* serotype 4b, isolated from dry sausage, and 1/2a, isolated from salami. All strains were preserved in glycerol at −20°C before use, except for the *C. jejuni* strains, which were stored at −80°C in defibrinated horse blood (E&O Laboratories, Ltd., Bonnybridge, Scotland). *Campylobacter jejuni* strains were cultivated in Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) lysed defibrinated horse blood under microaerobic conditions (6% CO_2, 6% H_2, 4% O_2, 84% N_2), while other bacterial strains were cultivated in tryptic soy broth (TSB) (Bio-Rad, Hercules, CA). For all experiments, all strains were cultivated at 37°C until the stationary growth phase was reached. On the basis of growth curve parameters (data not shown), bacteria were suspended and diluted in HS buffer to ca. 5×10^7 CFU/ml. To determine the exact number of viable, cultivable bacteria used in the experiments, serial dilutions were plated on PCA and incubated for 48 h at 30°C, except for *Campylobacter*. For the latter, suspensions were plated on Mueller-Hinton agar (MHA) (Oxoid, Basingstoke, United Kingdom) followed by 48 h of incubation at 42°C under microaerobic conditions.

Coculture experiments. All coculture experiments with *A. castellanii* and the 10 foodborne bacterial strains were performed in HS buffer at 25°C. All media and products used during the assays were acclimatized to the desired temperature before the start of the experiments. First invasion/uptake assays were performed to evaluate the bacterial invasion/uptake efficiency by amoebal trophozoites. Then encystment monitoring assays were performed to assess whether the presence of bacteria influenced the kinetics of the encystment process and *vice versa*. Finally long-term intracystic survival assays and transmission electron microscopy (TEM) analysis were carried out to assess the presence and viability (after excystment) of bacteria inside cysts at different time points.

Invasion/uptake assays. Invasion/uptake assays were carried out as described previously (26, 36) with small modifications. Culture flasks (25-cm^2) were seeded with *Acanthamoeba castellanii* trophozoites (10 ml/flask at a concentration of ca. 5×10^5 cells/ml HS buffer) and incubated at 25°C for 1 h to allow amoebal settlement and adhesion prior to infection. The medium was gently removed, and 10 ml bacterial suspension at a concentration of ca. 5×10^7 CFU/ml HS was added to each flask to obtain a coculture with a multiplicity of infection (MOI) of ca. 100 bacteria per amoeba. Bacterial and amoebal monocultures were set up as controls. After 30 min of (co)cultivation at 25°C, cells were washed with HS buffer and treated with gentamicin sulfate solution (Sigma-Aldrich, St. Louis, MO) at a final concentration of 100 μg/ml HS buffer for 2 h at 25°C to kill extracellular bacteria. The amoebae were then washed with HS buffer to remove the gentamicin and lysed with 0.5% sodium deoxycholate for 5 min to recover intra-amoebal bacteria. This treatment was effective (100%) in lysing amoebal trophozoites without affecting bacterial viability (data not shown). As during the invasion/uptake assays, amoebae were incubated in HS buffer for only a short time: no conversion to cysts took place. Cell suspensions of coculture and amoebal monoculture control setups were plated on MHA (*Campylobacter*) or PCA (*Salmonella*, *Escherichia*, *Listeria*, or *Yersinia*) as described above to determine viable intra-amoebal CFU counts.

Encystment monitoring assays. *Acanthamoeba* trophozoites were infected with bacteria (MOI of 100:1 in HS buffer) as described for the invasion/uptake assays and incubated at 25°C for 6 days. Amoebal and bacterial monocultures in HS buffer were set up as controls. The encystment of *Acanthamoeba* was verified with a light microscope. At day 0 (d0) and on d1, -2, -3, -4, and -6, viable trophozoites and cysts were counted by trypan blue exclusion assays in a Fuchs-Rosenthal counting chamber. The number of mature cysts was determined by lysis of trophozoites and immature cysts by 3% HCl (28, 37). The number of extra-amoebal bacteria during encystment and of bacteria in the monoculture controls was determined by plating serial dilutions on MHA or PCA, as described above.

Long-term intracystic survival assays. To assess if bacteria were able to survive inside amoebal cysts for longer periods (days to weeks), long-term intracystic survival assays were carried out (Fig. 1). Cocultures (combinations of amoebae with a specific bacterial strain) and amoeba and bacterial monoculture controls were set up as described above. Separate culture flasks were used for each excystment time point (described below). After 6 days of incubation in HS buffer at 25°C, cells were treated overnight at 25°C with 3% HCl (pH 0.2) to kill extracellular bacteria, trophozoites, and immature cysts. The efficiency of HCl at killing trophozoites was verified by light microscopy. Cells were then washed with PAS buffer and treated with gentamicin sulfate (100 μg/ml in HS buffer) for 2 h at 25°C to kill any remaining viable extracellular bacteria. Subsequently cysts

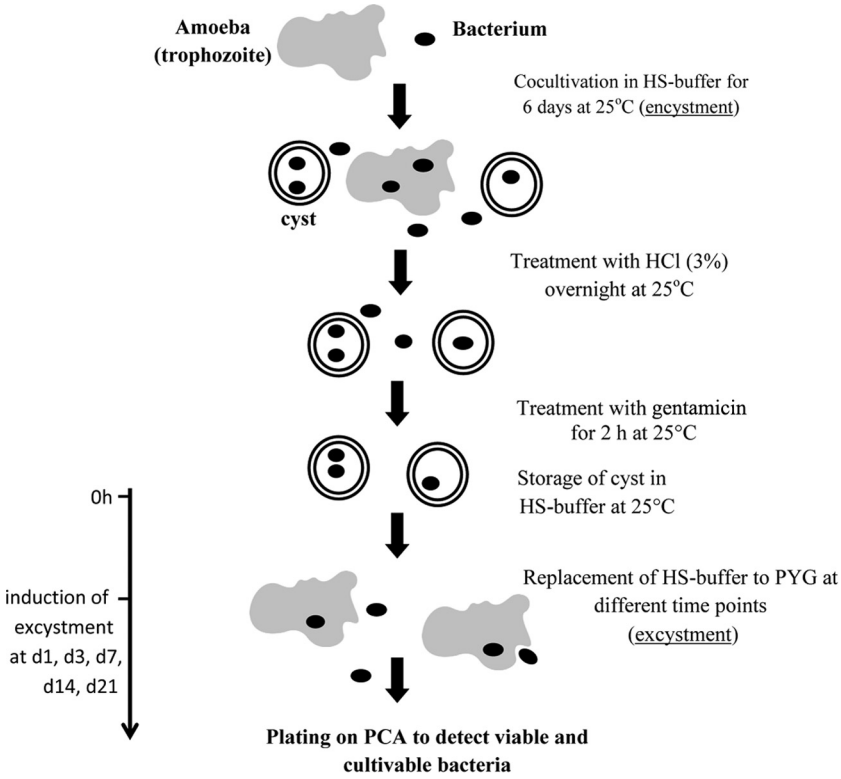


FIG 1 Overview of the experimental setup of the intracystic survival assay.

were washed, resuspended in fresh HS buffer, and incubated at 25°C until the time point for induction of excystment. For the latter, at day 0 (i.e., immediately after the gentamicin treatment) and after 1, 3, 7, 14, and 21 days, the cells were washed with PAS and then incubated at 25°C in nutrient-rich PYG medium as the excystment medium (26). During excystment, internalized bacteria are released into the medium, where they can further replicate. The process of excystment in both cocultures and amoebal controls and the presence of released bacteria in the coculture flasks and of viable bacteria in the bacterial control flasks were checked by light microscopy. Exact quantification of recovered bacteria was assessed by plating on MHA or PCA, as described previously. Enrichment cultures were set up to detect stressed bacteria and/or low bacterial concentrations (enrichment in TSB, ½ Fraser broth for *L. monocytogenes*, and MHB plus 5% [vol/vol] horse blood for *C. jejuni*, followed by plating on MHA or PCA). The identity of the recovered bacteria was confirmed by conventional biochemical testing (ISO6579-FDAmd1, ISO10273, ISO10272-1, ISO11290-1/A1, and ISO16654; www.iso.org).

TEM. To determine the presence and exact subcellular location of intracystic bacteria, mature cysts, obtained after HCl and gentamicin treatment (see above), were prepared for observation by transmission electron microscopy (TEM). Chemical fixation was performed on collagen-coated inserts as described by Lambrecht et al. (38). Briefly, at d0 and on days 3 and 14, cysts were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. Subsequently, cells were rinsed with sodium cacodylate buffer, postfixed with osmium tetroxide, dehydrated with ethanol, and embedded in Spurr's medium. Sections were stained with uranyl acetate and lead citrate and studied with a Jeol JEM-1010 transmission electron microscope (Jeol, Ltd., Tokyo, Japan) operating at 60 kV. Pictures were digitized using a Ditabis system (Pforzheim, Germany). Per image section, 30 cysts were studied to obtain an indication of the infection ratio (percentage of cysts with intracellular bacteria) and infection intensity (mean number of intracellular bacteria inside bacterium-harboring cysts). Identification of bacteria was based on size and the presence of a double surrounding membrane.

Data analysis. All experiments were repeated at least three times. Qualitative and quantitative data were recorded in an Excel spreadsheet, and statistical analysis was performed on the quantitative data using the software SPSS version 21 (IBM Corp., Armonk, NY).

For the invasion/uptake assays, a Wilcoxon rank sum test was used to detect differences in entry efficiency between and within species. For the latter, strains of the same bacterial species were clustered. For the encystment monitoring assays, Wilcoxon rank sum tests were performed to (i) compare the percentages of cysts between cocultures and amoebal monocultures at each time point and (ii) to compare the absolute numbers of viable extracellular bacteria between cocultures and bacterial monocultures during encystment.

RESULTS

Foodborne pathogenic strains enter *Acanthamoeba castellanii* with different efficiencies. Invasion/uptake assays were performed to evaluate the bacterial invasion/uptake efficiencies by amoebal trophozoites before encystment. The results demonstrate that foodborne bacterial pathogens enter the *Acanthamoeba* trophozoite with various efficiencies (Fig. 2). No viable intraamoebal listeriae and campylobacters were recovered after 30 min of cocultivation in HS buffer at 25°C. The invasion/uptake efficiency of the other tested bacteria ranged from 0.0013% to 0.0306%. Significant differences in entry efficiency were observed between but not within species ($P > 0.05$). *Escherichia coli* ($P = 0.002$) and *S. enterica* ($P = 0.002$) exhibited significantly higher invasion/uptake by the amoebae than *Y. enterocolitica*. Indeed, after 30 min of cocultivation, the invasion/uptake index for both *E. coli* and *S. enterica* was ca. 1 intracellular bacterium/amoeba,

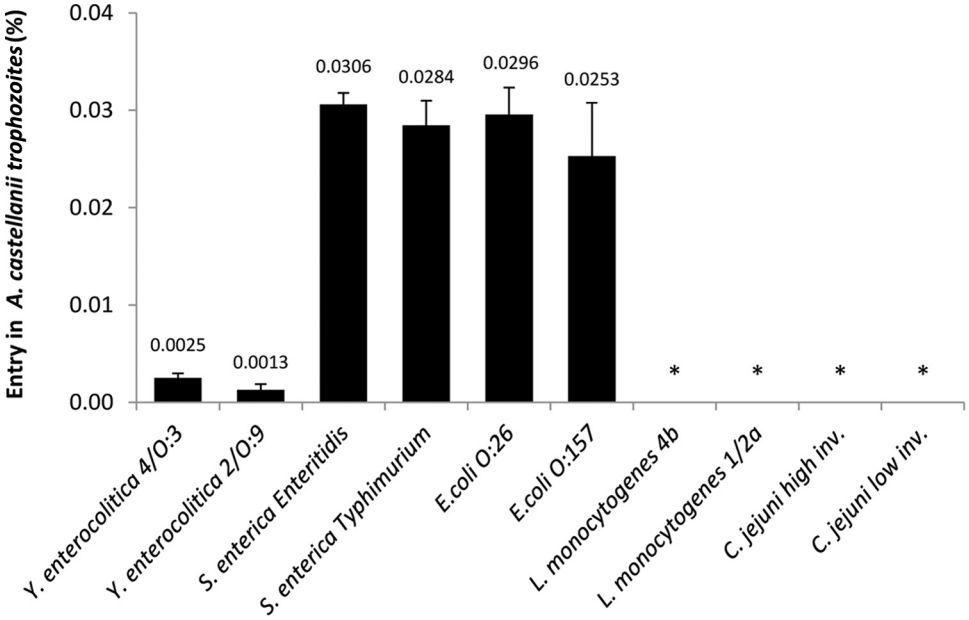


FIG 2 Entry efficiencies of different strains belonging to five foodborne bacterial pathogen species in *A. castellanii* trophozoites. Shown are the percentages of internalized viable, cultivable bacteria related to the initial inoculum after 30 min of cocultivation with *A. castellanii* in HS buffer at 25°C. Bars represent the mean ± standard error from four replicate experiments. *, no viable bacteria could be recovered (detection limit of 1 CFU/ml). inv., invasiveness towards Caco2 cells.

whereas for the yersiniae, this value was only 0.05 intracellular bacterium/amoeba.

The presence of bacteria has no impact on amoebal encystment, except with *Y. enterocolitica*. *Acanthamoeba* is known to encyst in response to adverse environmental conditions, including food shortage. To assess if the presence of bacteria, being a potential food source for amoebae, affects the encystment process, encystment monitoring assays were performed. In general, encystment started within 24 h of incubation in HS buffer. After 2 days, ca. 54 to 93% of the trophozoites, in both cocultures and amoebal monoculture controls, had converted to immature cysts. After 6 days, all cysts were mature. With the exception of *Y. enterocolitica* strain 2/O:9 (Fig. 3), no significant differences between cyst percentages in cocultures and monoculture controls could be observed ($P > 0.05$). In the presence of *Y. enterocolitica* 2/O:9 (Fig. 3B), cyst percentages were significantly lower than in amoebal monocultures for all time points ($P < 0.05$). On day 6, only 71% ± 5% (mean ± standard error of the mean [SEM]) of the amoebae were encysted when cocultivated with *Y. enterocolitica* 2/O:9, in comparison to 95% ± 2.5% under the control condition ($P = 0.028$).

In general, the presence of encysting amoebae had no significant influence on the viability of the bacteria ($P > 0.05$) (data not shown). After 6 days of cultivation, the number of viable extra-amoebal bacteria in both cocultures and bacterial monocultures was still ca. 7 log CFU/ml, except for campylobacters. For the latter, no viable extra-amoebal bacteria could be recovered after 3 days in either cocultures or bacterial monocultures.

Intracystic survival of foodborne pathogens. The recovery of viable internalized foodborne pathogens from *Acanthamoeba* cysts after periods of days to weeks was species, strain, and time dependent (Fig. 4). In general, *S. enterica*, *L. monocytogenes*, *Y. enterocolitica*, and *E. coli* could be recovered after induction of excystment, indicating that they were able to survive inside *A. castellanii* cysts. In contrast, cultivable *C. jejuni* cells could never be retrieved after induction of excystment.

Viable *S. enterica* and *L. monocytogenes* cells could be recovered from cysts after 21 days and 14 days (i.e., 27 and 20 days after the setup of the encystment treatment), respectively. In the bacterial monoculture controls, no bacteria were detected, confirming that the HCl and gentamicin treatments, together with the washing steps, were effective in removing bacteria. Moreover, in none of the cocultures could bacteria be recovered within 24 h after the replacement of the HS buffer with PYG medium, indicating that the bacteria were inside the cysts and not attached to the outer surface of the cyst wall. After 24 to 48 h in PYG medium, excystment occurred and trophozoites and (extracellular) bacteria were visible. After excystment, the extracellular *S. enterica* and *L. monocytogenes* cells grew rapidly, and after 3 days, concentrations of up to 9 log CFU/ml were reached. When the bacteria became too dense, the amoebae started to round up, lysed, or formed cysts again (data not shown).

Yersinia enterocolitica strains could be recovered from amoebal cysts at excystment time points d0, d1, and d3 by plating on PCA. At later excystment time points, no yersiniae could be detected.

Escherichia coli survived for only a limited time inside cysts. Strain O:26 could be recovered until d3, whereas strain O:157 could only be recovered at d0 (i.e., 6 days after the initial experimental setup). Three days after induction of excystment, viable extracellular *E. coli* O:26 and O:157 were observed, and from day 5 onwards, extracellular *E. coli* concentrations of 8 log CFU/ml were detected. The trophozoites began to lyse during the first day following excystment (data not shown). However, at later excystment time points (d7, d14, and d21), lysis of trophozoites decreased, amoebal trophozoites remained intact, and no viable *E. coli* cells could be recovered.

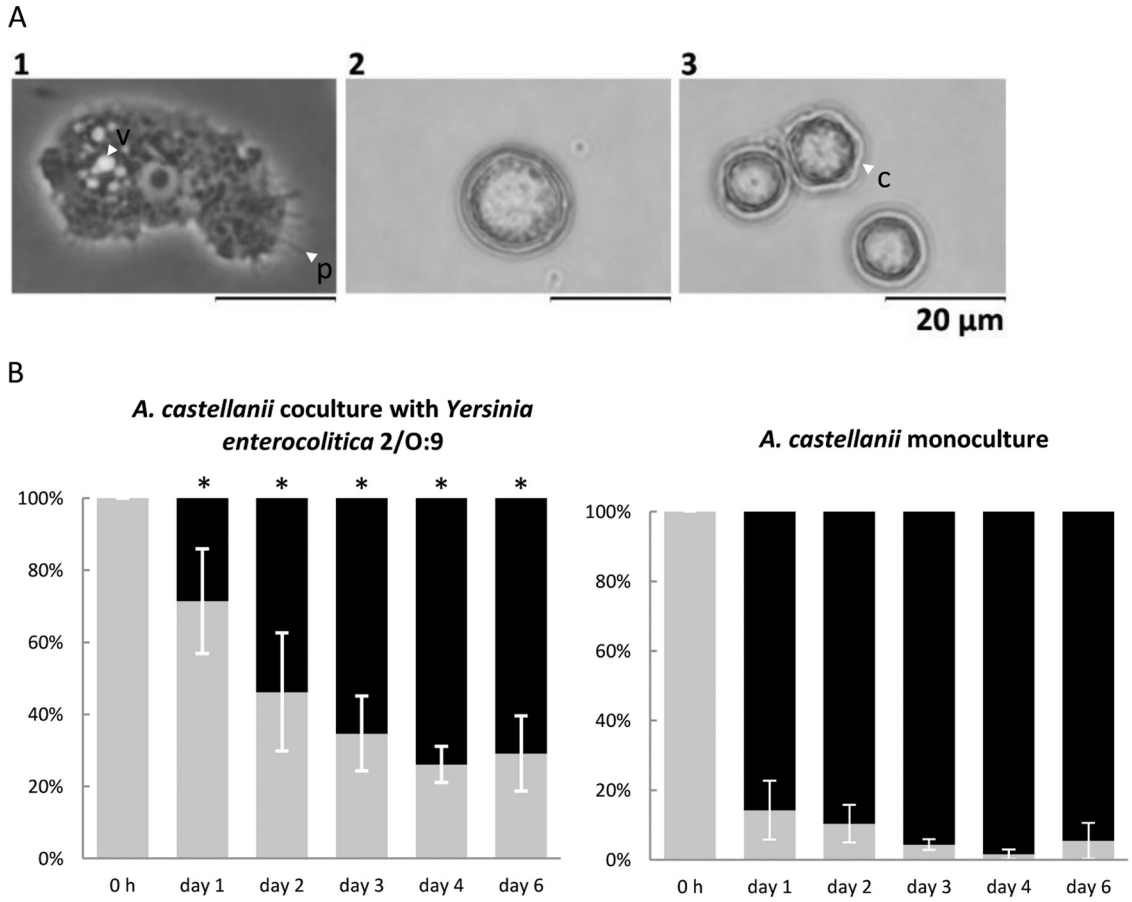


FIG 3 (A) Encystment process of *A. castellanii*; (B) *Yersinia enterocolitica* strain 2/O:9 inhibits encystment of *A. castellanii*. (A) *A. castellanii* trophozoite (panel 1), immature cyst (panel 2), and mature cyst (panel 3). Note the presence of pseudopodia (p) and vacuoles (v) in panel A1 and the thick double-layered cell wall (c) in panel A3. (B) Percentages of *A. castellanii* trophozoites (gray) and (im)mature cysts (black) in the total amoebal count when cultivated in the presence (coculture) or absence (monoculture) of *Y. enterocolitica* at 25°C in HS buffer. Bars represent the mean ± standard error from four replicate experiments. *, significant difference ($P < 0.05$) between the means of coculture and the corresponding monoculture condition.

No viable, cultivable *C. jejuni* cells were recovered from *A. castellanii* cysts at any time point after induction of excystment, either by direct plating or by enrichment, although TEM images demonstrate the presence of intact intracystic bacteria at d0 and d3 (Fig. 5E).

The viability of extracellular *C. jejuni* already decreased during the encystment stage, as after 2 days of incubation in HS buffer, extracellular *C. jejuni* cells were coccoid and nonmotile in both cocultures and monocultures.

Foodborne pathogens localize inside the cyst cytosol. Transmission electron microscopy was performed to determine the exact intracystic location of the bacteria and to obtain an indication of the infection intensity and ratio. All tested foodborne bacterial pathogens were located in the amoebal cytosol and were not visibly surrounded by an amoebal vacuolar membrane (Fig. 5). At time point 0 h, the percentage of infected cysts (infection ratio) ranged from 17% to 53%, and the mean number of intact bacteria inside cysts (infection intensity) varied between 1.4 and 2.6 (Table 1).

DISCUSSION

Our study proves that four of the most frequently reported foodborne bacterial pathogens (*S. enterica*, *L. monocytogenes*, *E. coli*, and *Y. enterocolitica*) can survive encystment and excystment by the ubiquitous amoeba *Acanthamoeba castellanii* and can persist inside the cyst cytosol. While species-specific differences in trophozoite invasion/uptake efficiencies were observed, these could not be correlated with the pronounced differences in intracystic survival periods, ranging from d0 for *E. coli* O:157 up to d21 for both *S. enterica* strains. Moreover, intracystic bacteria were found to be protected against hostile environmental conditions, in this case antibiotic treatment (100 μg/ml gentamicin) and low pH (pH 0.2). For all tested species, including *C. jejuni*, the presence of intracystic bacteria was confirmed by transmission electron microscopy. Up to 53% of the cysts contained foodborne pathogenic bacteria. After induction of excystment, *S. enterica*, *L. monocytogenes*, *E. coli*, and *Y. enterocolitica* were released or triggered their release into the environment and were able to grow successfully. Bacterial release into the environment can occur through expulsion of bacterium-containing pellets or vesicles from intact amoebal cells or through amoebal lysis (3). The results of the present study could not provide a conclusive answer on the release mechanisms used by the tested bacteria. No culturable *Campylobacter* bacteria could be recovered from cysts after excystment. Hence the identity, viability, and culturability of the detected intracystic struc-

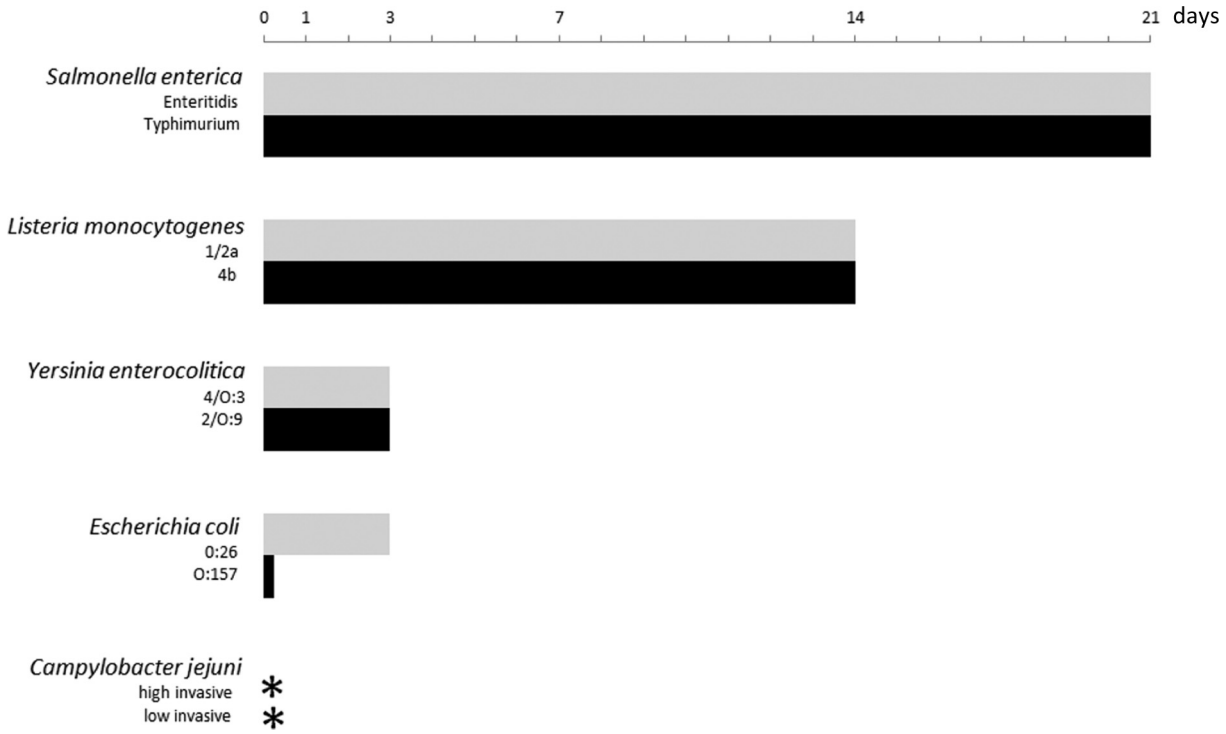


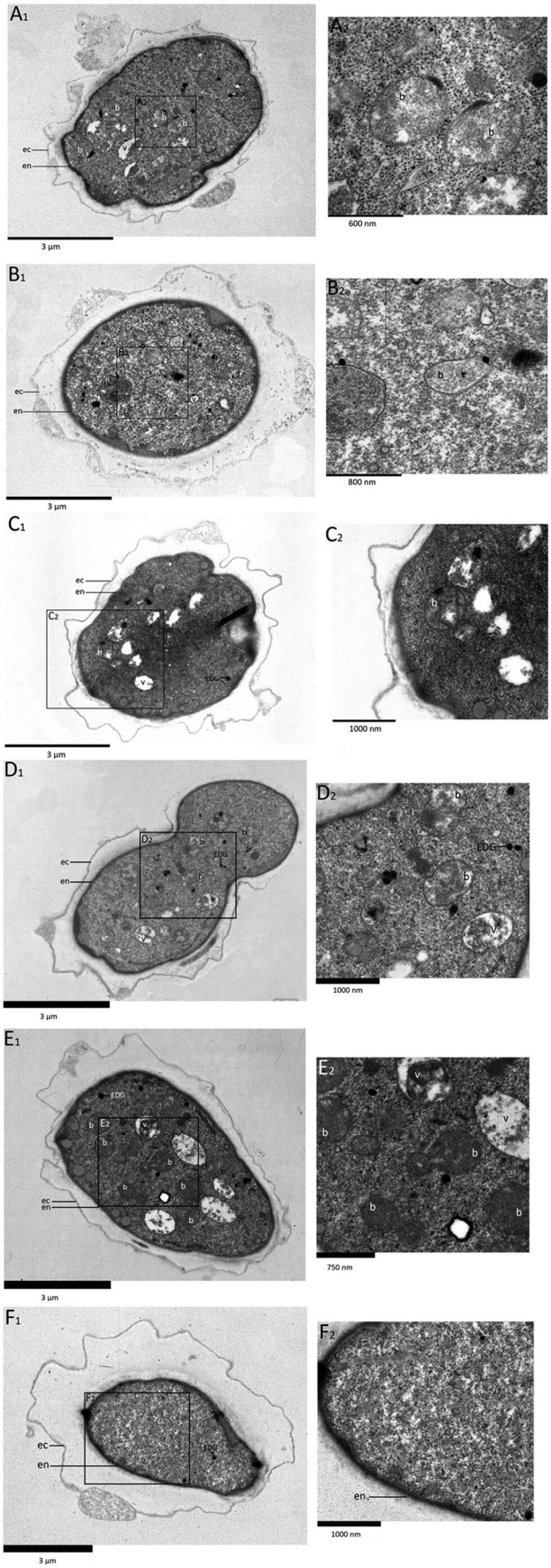
FIG 4 Survival (*S. enterica*, *L. monocytogenes*, *Y. enterocolitica*, and *E. coli*) or presence (*C. jejuni*) of foodborne pathogen strains inside *A. castellanii* cysts. Ten strains belonging to five foodborne pathogenic bacterial species were cocultivated with *A. castellanii* trophozoites (MOI of 1:100 in HS buffer at 25°C). After 6 days, cysts were treated with HCl and gentamicin and stored in HS buffer at 25°C ($t = 0$). To detect the intracellular survival of bacteria, excystment was induced at different time points (d0, d1, d3, d7, d14, and d21) by replacing HS buffer with nutrient-rich medium. Viability of bacteria was confirmed by colony counting. Bars represent the presence of viable, cultivable bacteria after excystment ($n \geq 3$). For each species, two strains were tested, as indicated by the black and gray bars. *, presence of bacteria inside cysts (as determined by transmission electron microscopy), which were not cultivable after excystment.

tures by TEM could not be confirmed. Our results thus show that protozoan cysts can provide internalized foodborne pathogenic bacteria with a shelter against adverse environmental conditions (21, 25). This sheltering could have significant repercussions on the ecology and epidemiology of foodborne pathogenic bacteria.

To our knowledge, no research has yet been performed to determine the survival capacities of *Salmonella* species in protozoan cysts. We show that *S. enterica* can survive for at least 3 weeks inside *A. castellanii* cysts, and preliminary results suggest that they might even survive for up to 3 months inside the cysts (data not shown). Viable *L. monocytogenes* cells could be recovered from cysts for up to 2 weeks. In an earlier study, no viable *L. monocytogenes* cells could be recovered from *A. castellanii* cysts after 34 days, suggesting that internalized bacteria, if present, may have died within that time frame (39). Moreover, survival inside cysts was shown to depend on the *Listeria* strain and *Acanthamoeba* species used (40). Previous experiments demonstrated survival of *L. monocytogenes* in cysts of *Colpoda* spp. (41) and *Tetrahymena pyriformis* (42). However, some are skeptical about the latter observation as *Tetrahymena pyriformis* is not known to form cysts (3).

Y. enterocolitica and *E. coli* only survived for up to 3 days inside *A. castellanii* cysts. As 2 days after inoculation 90% of the amoeba were already encysted, bacteria recovered at the arbitrary d0 time point (6 days after initial inoculation) were likely to have been encapsulated in the cysts for at least 5 days. No cultivable *Y. enterocolitica* and *E. coli* cells could be recovered from the cysts at time points later than d3. Transmission electron microscopy confirmed that there were no intact intracystic bacteria present at later time points. This indicates that *Y. enterocolitica* and *E. coli* invade or are taken up by amoebae (cf. confirmed by invasion/uptake assays) and survive for only a limited period of time inside amoebal cysts. Failure to persist for extended periods of time is probably due to intracystic nutrient depletion. The intracystic environment is not an optimal environment for bacteria as during cyst formation, excess food and water are expelled and the protozoon condenses (43). No data are yet available in the literature regarding intracystic survival of *Y. enterocolitica*. Matin and colleagues suggested that survival of certain invasive, non-foodborne *E. coli* strains inside *Acanthamoeba* cysts was possible up to 43 h cocultivation under encystment conditions (30). As long-term intracystic survival was not tested in this study and a different amoebal encystment method was used, their results cannot reliably be compared with ours.

In contrast to the other tested foodborne pathogens, viable and cultivable *C. jejuni* strains could not be recovered after excystment. A previous study also briefly reported that no *C. jejuni* cells could be observed in *A. castellanii* cysts (44). However, in TEM images in the present study, intracellular structures that look like bacteria with an intact cell membrane were visible in *A. castellanii* cysts. We hypothesize that these structures are bacteria are in a viable but not cultivable state and cannot be recovered after excystment by plating on MH agar plates or by enrichment in MH broth. Another explanation could be that the intracystic campy-



lobacters do not survive the excystment process and are rapidly digested by the emerging amoebal trophozoites.

Determination of the exact intracystic location of bacteria was established by transmission electron microscopy. The TEM images of mature cysts at different excystment time points showed that all tested foodborne pathogenic strains were located inside the cyst cytosol. None of the investigated amoebal cross sections contained bacteria within the double cyst wall. Although these results are novel, bacterial entrapment in the cyst cytosol has already briefly been described before for *Legionella pneumophila* (25), *Vibrio mimicus*, and *Simkania negevensis*. The last two species could also be detected within the double cyst walls of *Acanthamoeba polyphaga* cysts (29, 32).

The species- and strain-specific differences in intracystic survival could not be related to bacterial invasion/uptake efficiency. Remarkably, no viable, cultivable *L. monocytogenes* or *C. jejuni* cells could be detected inside trophozoites after 30 min of cocultivation in HS buffer, although they could be detected inside cysts at different time points. The results of the invasion/uptake assays indicate that *L. monocytogenes* and *C. jejuni* prevent their uptake by the trophozoites or were not able to invade them within 30 min. Since later in the excystment process, viable intracystic bacteria were detected by either cultivation or TEM, it seems that those pathogens indeed need a longer invasion/uptake time. Contradictory information is published about the survival of *Campylobacter* and entry and survival mechanisms of *Listeria* in *Acanthamoeba* (for a complete overview, see reference 3). Differences can be attributed to variations in coculture assays (e.g., medium, temperature, and multiplicity of infection) and organisms (bacterial strains and protozoan species and strains) (3).

Initially, at least 17% up to 53% of the cysts were infected with foodborne pathogens, with the minimal estimated infection intensities ranging from 1.4 to 2.6 bacteria per infected amoeba. Moreover, after induction of excystment, all tested bacterial pathogens, with the exception of *Campylobacter*, were able to grow. Currently, the presence of free-living protozoa in food processing plants is not monitored as they are regarded as harmless. Our results, however, suggest that protozoan cysts could be a source of bacterial persistence in the environment and a route for host infection.

Previous studies have shown that bacteria in trophozoites (e.g., see references 45 and 46) and cysts (25) were better protected against biocides. This study confirms that in contrast with the free-living bacteria (cf. bacteria monoculture controls of the intracystic survival assays), encapsulation of foodborne bacteria inside protozoal cysts protects them against external stresses, including gentamicin treatment (100 μg/ml) and extreme low pH (pH 0.2, achieved by 3% HCl treatment). Protection of intracystic bacteria against gentamicin, a commonly used antibiotic in food animal production (47), has been reported previously and was attributed to the fact that the antibiotic could not pass the double cyst wall (32). The sheltering capacity of protozoan cysts therefore

FIG 5 TEM micrographs of *A. castellanii* cysts with internalized bacteria (A to E) and an amoebal monoculture control (F). (A) *S. enterica* serovar Enteritidis at d0. (B) *L. monocytogenes* 1/2a at d14. (C) *E. coli* O:26 at d0. (D) *Y. enterocolitica* 2/O:9 at d0. (E) *C. jejuni* highly invasive toward Caco2-cells at d0. b, bacteria; ec, ectocyst; en, endocyst; EDG, electron-dense granule; n, condensed nucleus; v, vacuole.

TABLE 1 Quantification of foodborne pathogenic bacteria inside *A. castellanii* cysts[a]

Species	0 h		d14	
	Infection ratio (%)	Infection intensity (no. of bacteria/cyst)	Infection ratio (%)	Infection intensity (no. of bacteria/cyst)
Salmonella enterica				
Serovar Enteritidis	53	2.6	47	1.4
Serovar Typhimurium	50	2.4	47	1.5
Listeria monocytogenes				
1/2a	37	1.6	27	1.4
4b	40	2.2	47	1.2
Escherichia coli				
O:26	23	1.4	ND[b]	ND
O:157	17	1.4	0	0
Y. enterocolitica				
4/O:3	40	1.6	ND	ND
2/O:9	40	2.1	0	0
Campylobacter jejuni				
High invasiveness	40	2.4	ND	ND
Low invasiveness	37	2.0	ND	ND

[a] The data are the result of counts of intact bacterial cells in 30 cysts in one cross section. As such, they only give an indication of the infection ratio (percentage of infected cysts) and infection intensity (mean number of bacteria inside the infected cysts' section).
[b] ND, not determined.

has important food safety and public health implications and could explain why pathogenic bacteria persist in food processing environments, despite thorough disinfection protocols and hygiene monitoring.

The results from the encystment monitoring assays demonstrate that the viability and the encystation process of the amoebae were not markedly affected by intracellular bacteria, except for strain *Y. enterocolitica* 2/O:9. For this strain, a significant delay in encystment was observed. This may be due to the fact that a portion of the bacterial population can be used as a food source, making the amoebae less prone to encyst. Another explanation could be that the encystment delay is a result of direct contact between amoebae and bacteria or is mediated by secreted factors. The influence of secreted factors on amoebal encystment has been demonstrated before, as a previous study reported that *Francisella tularensis* caused rapid amoebal encystment by factors secreted by amoebae and/or *F. tularensis* (26). It is also demonstrated that certain endosymbionts can prevent cyst formation of *Acanthamoeba* and *Hartmanella* (48).

In conclusion, we here show that the most common and widespread foodborne pathogenic bacteria can survive in the cytosol of free-living protozoan cysts for extended periods of time. Intracystic survival is associated with increased resistance to adverse environmental conditions (including antibiotics and low pH). Pathogenic bacteria may therefore also use cysts as a vector to colonize new habitats or to infect animal hosts and humans. Moreover, bacteria inside cysts are likely to be undetected by the standardized cultivation protocols for the detection of pathogenic bacteria in food-related environments. Further research is necessary to identify the factors and conditions that may prevent uptake of foodborne pathogens by free-living protozoa and subsequent protozoan encystation in order to decrease bacterial survival and persistence in food-related environments.

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The authors declare that they have no conflicts of interest.

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